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Field Test of Transgenic Cottons Containing a Gene from *Bacillus thuringiensis*

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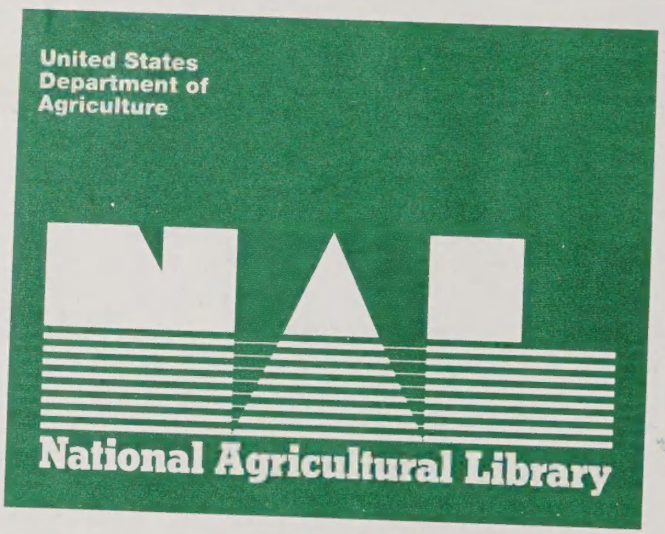
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Final Technical Report
Cooperative Research and Development Agreement No. 58-32U4-9-77
Between ARS and Agracetus Inc. (W. R. Grace and Co.)

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October 18, 1990

We evaluated four transgenic cotton strains, the parental strain and an adapted cultivar for resistance to tobacco budworm (TBW). The gene construction in each, derived from Bacillus thuringiensis Kurstaki (B.t.), did not produce enough toxin to provide an adequate level of protection to field grown plants. We did not detect any major effect on survival or growth of TBW or cabbage looper larvae in laboratory assays. Yield of each transgenic was lower than the parental strain, Coker 312 (C 312) and mean of seed cotton yields of all transgenics was significantly (0.06%) lower than C 312. Boll and seed weights for mean of all transgenics was significantly lower than C 312. Because we measured important and consistent differences between populations derived from independent transformation events, this suggests we may have to conduct yield, quality and performance trials for each transgenic strain as we use this technology. A biological sink of border rows was used to monitor and contain pollen transfer by insect from transgenic plants. Border rows showed a consistent and significant reduction in pollen dissemination as distance from test plot increased. Percentage of outcrossing decreased from 5% to less than 1% at 7 meters away from the test plot. A low level of pollen dispersal of less than 1% continued to sporadically occur to a distance of 25 meters. The border rows fulfilled their purpose of serving as a pollen sink to significantly reduce pollen dissemination from the test plot. Two manuscripts were written.

ABSTRACT

Genetically engineering plants to resist pest insects could be a major new tool in pest management practices for cotton, Gossypium hirsutum L. We evaluated four transgenic strains grown in field plots and compared their performance with their near isogenic, non-genetically engineered, parent cultivar, Coker 312 (C 312) and a locally adapted cultivar. Agronomic performance, fiber properties, and resistance to artificial infestation with first instar tobacco budworm (TBW), Heliothis virescens Fab., were evaluated. Using laboratory insect feeding assays, we also tested the ability of TBW and cabbage looper (CL), Trichoplusia ni Hubner, to survive on leaves, squares, and reproductive structures. This gene construction, derived from Bacillus thuringiensis Kurstaki (B.t.), did not produce enough toxin in the desired plant organs to provide an adequate level of protection to field grown plants. Neither did we detect any major effect on survival or growth of TBW or CL larvae in our laboratory assays. Yield of each transgenic was lower than C 312 and the mean of seed cotton yields of all transgenics was significantly lower than C 312 (at the 0.06 % alpha level). Boll and seed weights for the mean of all transgenics was significantly lower than C 312. Small differences in yield components and some fiber properties were detected in some of the transgenic strains. Our field and laboratory results show this particular B.t. gene construction did not provide enough protein to improve resistance of cotton plants to TBW. Because we measured important and consistent differences between populations derived from independent transformation events, this suggests we may have to conduct yield, quality, and performance trials for each transgenic strain as we use this technology.

U.S. cotton growers spend more than $200 million annually on chemical insecticides to control insect pests of cotton, Gossypium hirsutum L., which is the largest single crop market for U.S. insecticide sales. About one-third of the insecticides are used to control pests in the Lepidoptera. Successful development of genetically engineered, insect-resistant cotton plants could lead to the availability of a major new tool in pest management practices. Plants resistant to certain insect pests, could provide better protection, reduce production costs, and provide a less risky means of insect control. Use of this type of insect control also should benefit the environment through reduced use of chemical insecticides.

Agracetus, Inc., Middleton, WI, has pursued the development of transgenic cotton plants resistant to major lepidopteran insect pests. A modified bacterial gene from Bacillus thuringiensis Kurstaki HD-1 (B.t.), coding for the delta-endotoxin which is active against certain insect pests in the order Lepidoptera, was genetically engineered into a U.S. cultivar of cotton. A second bacterial gene from Klebsiella pnuemoniae which codes for neomycin phosphotransferase (NPT II), was used in conjunction with the B.t. gene as an in vitro selectable marker. The NPT II gene imparted resistance to the antibiotic kanamycin sulfate.

Deoxyribonucleic acid transfer into cotton was accomplished via the Agrobacterium tumefaciens Ti plasmid vector system. This vector system is non-pathogenic or "disarmed",i.e., all the crown gall disease genes normally found in the T-DNA were deleted. This system is also binary with the genes to be transferred on one plasmid and the genes encoding necessary functions for transfer, the "vir" genes, on a second

plasmid. Only genes delimited by the T-DNA borders on the transfer plasmid, in this case the B.t. and NPT II genes, were mobilized into the plant cell and integrated into the cell's genome. Barton et al. (1987) and Umbeck et al. (1987), described the construct used to transform cotton cells. All procedures used to insert the selectable marker, NPT II and B.t. genes into cotton were identical to those described by Umbeck et al. (1987).

Plants evaluated in the field test were from seed of self pollinated plants that had been analyzed previously in the laboratory of Agracetus. Insect-resistant cotton plants were characterized at the whole plant and molecular levels (P. Umbeck and K. Barton, unpublished data). The NPT II and B.t. genes were shown to be linked and inherited stably in a classical Mendelian fashion. In laboratory feeding trials with the cotton bollworm complex, Heliothis spp., and cabbage looper (CL) Trichoplusia ni Hubner, leaf tissue of the transgenic plants grown in pesticide-free environments reduced larval feeding activity, increased larval mortality, and prolonged the period from hatching to pupation (P. Umbeck and K. Barton, unpublished data).

The objectives of this investigation were to evaluate transgenic cotton plants grown under field conditions and compare their performance with a non-genetically engineered cultivar and with a locally adapted cultivar. Agronomic performance, fiber properties and resistance to artificial infestation with first-stage larvae of tobacco budworm (TBW), Heliothis virescens Fab., were evaluated.

MATERIALS AND METHODS

This experiment was conducted under the approval of the U.S. Department of Agriculture, Animal and Plant Health Inspection Service, Permit No. 88-351-13, Mississippi State University IBC Committee, and The Mississippi Department of Agriculture and Commerce. Research personnel made daily visits to the field test site and checked for signs of unauthorized entry and plant tampering. Only the personnel involved in the day to day operations of the research, Agracetus personnel, and regulatory authorities knew the exact location of the test plots.

Four transgenic cotton strains, TS 1, TS 2, TS 3, and TS 4, a non-transformed parental cultivar and a locally adapted cultivar were included in the experiment. The parental cultivar was Coker 312 (courtesy of Lloyd Langford, Seedco Corp., Lubbock, TX) and the locally adapted cultivar was DES 119, Bridge (1986). Strains TS 1 and TS 3 were derived from the same original regenerated plant, #3006. Strains TS 2 and TS 4 were obtained from regenerated plant, #3012. Regenerated plants #3006 and #3012 were products of independent transformation events, i.e., the foreign genes were integrated into different sites in the genome. In addition, strains TS 3 and TS 4 were bred to a homozygous condition for the NPT II/B.t. genes while strains TS 1 and TS 2 were segregating for the same foreign genes. All strains were planted 27 April 1989, in Marietta sandy clay loam (fine-loamy, siliceous, thermic Fluvaquentic Eutrochrepts) soil on the Plant Science Research Center at Mississippi State, MS. The plots were seeded at the rate expected to produce about 100,000 plants ha^{-1}. Plots were two rows 10 m in length and 1 m apart with a skip or non-planted row

between each two row plot. A 3 m non-planted alley was left between each two-row plot. Research plots were surrounded with border rows of cotton plants for 23 m on all sides to achieve biological containment of pollen. The border row plants were used to measure the extent of pollen transfer from transgenic plants in the research plots. To verify the extent of gene transfer to nearby cotton, seed samples were collected from each border row and will be tested for expression of the dominant selectable marker, NPT II. Results of this part of the field test will be reported elsewhere.

Seed were treated with Pentachloronitrobenzene before planting. At planting, Terrachlor super-x (5 Ethoxy-3-trichloromethyl-1,2,4-thiadiazole) at the rate of 11.2 kg ha^{-1} and aldicarb (2-methyl-2-(methylthio) propion-aldehyde O-(methylcarbamoyl)=oxime) at 0.34 kg a.i. ha^{-1} were applied in furrow. Plots were fertilized with 153 kg N ha^{-1} applied as 45 kg preplant, 72 kg, on 12 June and 36 kg, on 26 July. This amount of N was necessary due to leaching of N by excessive rainfall. Plants emerged under wet, cloudy, cool conditions. Plant stand was affected by the weather, but to the same degree in all research plots.

Field Experiments

The experimental design was a randomized complete block with six strains and six replications. Two experiments were planted side by side. Plots in experiment one were infested with 12 first instar TBW larvae per 13 cm of row applied four times, 7 d apart beginning on 15 June, using the techniques of Jenkins et al. (1982). Plots in the first experiment also were treated with weekly applications of azinphosmethyl or cythion to kill all major pest insects except TBW.

[illegible] 1 [illegible]

[illegible] plots were harvested [illegible]

[illegible] 23 [illegible]

The border row plants were [illegible]

[illegible] from treatments [illegible]

the extent of [illegible] transfer [illegible]

[illegible] from each border row and [illegible]

least detectable amount [illegible]

will be reported elsewhere.

Seed were treated with Pentachloronitrobenzene [illegible]

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23 [illegible] This [illegible] was necessary [illegible]

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[illegible]

[illegible]

Experiments

The experimental design was a randomized complete [illegible]

[illegible] four replications. Two experiments were planted [illegible]

[illegible] The [illegible] experiment was infested with 12 [illegible]

[illegible] per [illegible] supplied [illegible] beginning [illegible]

[illegible] [illegible] (1961) [illegible]

[illegible] treated with [illegible] applications

[illegible] all major [illegible] except [illegible]

This was designated the W/TBW treatment. The second experiment included the same cotton strains, but plots were not artificially infested with TBW and received full season insect control with the insecticides azinphosmethyl, cythion and fenvalerate. This was designated the W/O TBW treatment.

All plots were irrigated twice, 5 cm each on 4 and 11 Aug. Pix, mepiquat chloride, was applied at the rate of 1060 ml ha^{-1} on 3 Aug. Prep and DEF were applied at the recommended rate on 18 September. Plots were harvested with a two row mechanical harvester 2 weeks later.

We harvested 50 boll samples from each row of each plot in experiment two. After weighing, boll samples were ginned on a laboratory 10 saw gin and the data were used to determine boll weight (g seedcotton $boll^{-1}$), seed weight and lint percentage. Starlab, Knoxville, TN, measured the fiber properties. SAS, a commercial statistical package, was used to analyze the data by analysis of variance.

Laboratory Experiment

In addition, a third experiment for laboratory insect growth assays was planted within the border rows, adjacent to experiments one and two. In experiment three, single row plots 10 m long were planted of each cotton strain in a six replication randomized complete block experiment. Fertility, soil type, and planting methods and materials were the same as for the field plots of experiments one and two. No insecticides were applied to the plots in experiment three except for one application of nicotine sulphate for aphid control.

In the laboratory growth tests, larvae of TBW amd CL were grown on excised plant parts from field-grown plants. The TBW used were from our colony that is outcrossed each year to field collected male moths. The first generation following the cross is put into diapause and stored as pupae during the winter. In April of each year the TBW colony is started from these diapausing pupae. The CL were obtained from the USDA, ARS Western Cotton Research Laboratory, Phoenix, AZ. This insect strain had been in laboratory culture for many generations.

For the laboratory insect feeding studies, leaves, squares (flowerbuds) plus bracts, and reproductive structures (anthers and stigma) were picked in the field and transported to the laboratory in sealed plastic bags inside a cooler. Leaf discs 28 mm in diameter were cut from leaves and used for the feeding trials. Squares were evaluated with bracts intact. For the tests involving anthers, the anther column was cut from the base of the square so that only anthers and stigma were offered to the insects.

The rearing chambers for the insects were disposable plastic trays with 16 cells. Agar was poured into eight cells of the 16 cell tray, then plant parts were placed in the eight cells of the tray containing the agar. The remaining eight cells were left blank and functioned to stabilize the tray in the incubator. One first instar larva was placed in each cell and the tray was sealed with the perforated lid used in rearing (Davis et al., 1990). Eight cells were used in each replication and six replications were run for each tissue evaluated. Trays with the plant parts and larvae were placed in an incubator maintained at 28^{o}C.

After three days of incubation, the trays were removed and the number of live larvae was recorded. Larvae that had demonstrated feeding behavior by day 3 were transferred to a fresh piece of plant tissue. The trays were resealed and returned to the incubator. New plant tissue was placed in the trays every 2 to 3 d until the end of the test. Length of test varied with the tissue type and the insect feeding duration of the experiment. After each experiment, the insects from the eight cells in each replication were removed and weighed as a group. The number of insects and their total weight were recorded. All plant parts harvested, but not fed to insects were autoclaved before disposal. SAS was used to analyze the data.

All remaining seed cotton from the field test was harvested and ginned on a laboratory 10-saw gin and the seed were returned to Agracetus. Seedcotton remaining in the field after picking the plots and border rows was shredded and tilled into the soil to allow the seed to rot during the winter. The field was monitored the next spring and all seedlings were killed with a combination of soil tillage and herbicides.

RESULTS

Laboratory Experiment

Insect growth studies performed at Agracetus showed that, during an insect feeding trial of 10 to 15 days, older (fully expanded) cotton leaves displayed insect resistance. Therefore, we designed our laboratory experiments to test field-grown transgenic cotton plants in a similar fashion. Tobacco budworm larvae were grown on new leaves (2.5 to 3.0 cm dia) or old leaves (mature) for 13 d. After 13 d, larvae grown on old leaves of TS 1 and TS 4 were significantly smaller than those on TS 2 and TS 3 and on the non-transformed Coker 312 (Table 1). The weight of TBW larvae was reduced 15% on TS 1 and 17% on TS 4 compared to the parental cultivar Coker 312 (Table 1). Actual weight differences, however, were small and probably not biologically significant. No differences were found in the weight of larvae among transgenic lines and Coker 312 after 13 d on new leaves, although larvae grown on DES 119 were significantly smaller than on all other strains. No differences in survival were detected on new or old leaves.

Tobacco budworm larvae were grown on squares for 8 or 11 d with no differences in weight or survival between the transgenic strains and Coker 312. The TBW larvae grown for 13 d on anthers were considerably larger than those grown on any other plant tissue. However, there were no significant differences between any of the transgenic strains and Coker 312. Larvae grown for 13 d on anthers of DES 119 were significantly smaller than those grown on other strains. Thus, we did not detect any major effects on TBW larval weights or survival due to the insertion of the B.t. gene into cotton. The smaller larvae grown on new leaves and anthers of DES 119 compared to Coker 312 reflects the

progress made in breeding cotton cultivars to be more resistant to insect pests such as the TBW. This also shows that genetic resistance to TBW exists among cultivars of cotton.

The CL is a lepidopteran insect which is not a major pest of cotton but is more sensitive to the B.t. toxin than TBW. We therefore grew CL on old leaves for 13 d and measured survival and weight. No differences in larval weight were detected among the transgenic strains and Coker 312 (Table 1). While the survival showed a marginally significant reduction for line TS 1, we believe that this reduction was again biologically non-significant. Thus, with CL we observed no major detrimental effects of the B.t. gene's presence in cotton.

Our laboratory insect growth studies suggested that the B.t. gene did not produce sufficient toxin in the proper plant organs to provide a consistent level of protection in plants grown under field conditions. We observe no major effect on survival or growth of TBW or cabbage looper larvae. Therefore, we anticipated that the cotton plants in the field test would not be adequately protected from damage by artificial infestation of TBW.

Field Experiment: Resistance to TBW

The high level of infestation of TBW attained in the field plots resulted in a yield difference of 967 kg ha^{-1} (57%) in DES 119 (Table 2). We found no difference in resistance to TBW among the transgenic strains and Coker 312. DES 119 yielded significantly more lint than either the transgenic strains or Coker 312. The percent of potential yield, a relative measure of resistance, varied from 28.0 to 41.5%; however, the differences were not significant (Table 2). Thus,

genetically engineering the B.t. gene construct into Coker 312 did not improve its resistance to a high level of artificial infestation with TBW.

Field Experiment: Yield and Yield Components and Fiber Properties

In no case did the lint yield of any transgenic strain exceed the yield of C 312 and in most cases it was lower (Table 2). In a combined analysis over infested and sprayed plots, the contrast of the mean lint yield of TS 2 and TS 4 vs C 312 was significantly different at the 0.01 level; however the contrast of the mean of TS 1 and TS 3 vs C 312 was not significantly different. Thus, we detected a significant reduction in lint yield in populations regenerated from plant #3012, but not from plant #3006.

TS 1 and TS 3 had significantly higher lint percent than C 312; whereas, TS 2 and TS 4 were not different from C 312, (Table 2). Boll and seed weights were significantly lower on each transgenic strain than for C 312. The contrast of the mean of the transgenics vs C 312 was significant at the 0.01 level for both boll and seed weights. Thus, we detected a significant reduction in boll and seed weight in populations regenerated from both plant #3006 and plant #3012; however we detected a significant increase in lint percent in populations regenerated from plant #3006 but not from plant #3012.

It would appear from these studies that yield evaluation trials will be required on each transgenic strain as they are developed in the future. These changes in yield and yield components are major and are not in line with what some have said about inserting single genes into plant chromosomes and only affecting single properties. The basis for these differences could have arisen from several possible causes. The

two most important sources of variation would be the independent transformation events that gave rise to #3006 and #3012 which could result from positional effects on expression of important agronomic characteristics, pleitrophic effects of the inserted gene or some somaclonal mutational events that occurred during the tissue culture process.

Most fiber properties of the transgenic strains were not different than those of C 312 (Table 3). However, TS 2 produced significantly longer fibers, as measured by the 2.5% span length, than did C 312. The contrast of the mean 2.5% span length of TS 2 and TS 4 vs C 312 was significantly different at the 0.05 level.

For each of the yield components and fiber properties TS 1 and TS 3 were similar and TS 2 and TS 4 were similar, thus emphasizing the consistent response of populations regenerated from the same transformed plant.

DISCUSSION

In the world's first field test of cotton genetically engineered to express the delta-endotoxin form Bacillus thuringiensis we did not detect any effects of the gene on resistance to TBW. We did, however, show some important biological properties of these genetically engineered cotton strains. For each population the homozygous and heterozygous strains were similar in all properties we measured. This increases our confidence that the differences or lack of differences were real. The populations derived from transformed plant #3006 (TS 1 and TS 3) did not differ significantly from C 312 in lint yield, micronaire, fiber length, fiber elongation or fiber strength; whereas, they had significantly greater lint percentage smaller bolls and smaller seed than C 312. The populations derived from transformed plant #3012 (TS 2 and TS 4) showed significantly lower lint yield, smaller bolls and seed and longer fibers (2.5% span length), than C 312; whereas, lint percentage, micronaire, 50% span length, fiber elongation and fiber strength were not different from C 312. Thus, we measured important and consistent differences between the populations derived from independent transformation events. This may indicate that yield, quality and performance trials will be required for each transgenic strain as we use this technology.

It is not possible to decide whether the differences among transgenic strains and Coker 312 are due to positional effects, i.e., the site of chromosomal insertion of the foreign genes, or to somaclonal variation, i.e., plants obtained from a homogenous cell culture that exhibits stable or heritable differences. The effect of chromosome

position on the expression of foreign genes has been described by others (Barton et al., 1989; Jones et al., 1985). For example, it has been shown that a particular gene construct can vary in expression up to 200-fold at the whole plant level based on the site of insertion into the plant genome. However, the effects these changes have on agronomic performance have not been adequately characterized.

The possibility that somaclonal variation could serve as a cause for changes in agronomic performance has been well documented in other crops (Larkin and Scowcroft, 1981). More recently, the potential for such changes to occur in cotton has been shown (Stelly et al., 1989; Li et al., 1989). It would not be surprising if the effects that we observed were due to a combination of the insertion of foreign genes into the genome of Coker 312 and a carryover effect of the tissue culture process. It is important and encouraging to note that foreign genes and tissue culture did not have major adverse effects on fiber properties in these transgenic cotton strains. The smaller bolls and seed and the increase in fiber length and lint percentage could be considered as positive changes. The one negative effect was the significant reduction in lint yield of transgenic strains TS 2 and TS 4 compared with C 312.

It was disappointing that the expression of the B.t. gene was insufficient to cause the plants to resist the damaging effects of TBW. However, recent chimeric gene constructions have shown dramatic improvements not only in the production of the B.t. protein, but also in ability of the host plants to withstand damage by insect pests (Barton et al., 1989). While our first endeavor to develop a novel method of insect resistance in cotton was unsuccessful, as with any new

technology, the intricacies of genetically engineering host plants to resist insects will require further refinements in our understanding of gene regulation at the molecular and whole plant levels.

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Table 1. Weight and survival of tobacco budworm (TBW) and cabbage looper (CL) grown on various plant parts in the laboratory.

Cotton strains	TBW † 13 d old leaves Wt	TBW ‡ 13 d new leaves Wt	TBW ‡ 13 d new leaves Survival	TBW § 8 d square Wt	TBW § 11 d square Wt	TBW § 13 d anthers Wt	TBW § 13 d anthers Survival	CL § 13 d old leaves Wt	CL § 13 d old leaves Survival
	-mg-	-mg-	---%----	-mg-	-mg-	-mg-	---%----	-mg-	---%----
TS1	115	131	88	36	130	308	86	130	74
TS2	142	118	87	32	130	298	91	131	89
TS3	125	130	87	30	130	299	93	123	99
TS4	113	122	85	29	140	300	88	130	97
Coker 312	136	112	84	27	120	302	92	132	90
DES 119	135	84	85	26	137	248	93	136	87
LSD 0.05	21	14	6	10	28	37	15	12	16
F Test	*	*	NS	NS	NS	*	NS	NS	*

† Mean of 7 tests of 6 replications each with 8 larvae per replication.

‡ Mean of 4 tests of 6 replications each with 8 larvae per replication.

§ Mean of 1 test of 6 replications each with 8 larvae per replication.

Table 2. Yield components of transgenic cotton strains grown under field conditions.

Cotton Strains	Lint ha^{-1} W/TBW	Lint ha^{-1} WO/TBW	Percent of potential†	Lint percentage	Boll size	Seed index
	-------kg-------		-----%----	----%-----	--g--	--g--
TS 1	482	1588	30.2	39.47	5.96	10.42
TS 2	405	1488	28.0	38.00	5.25	10.35
TS 3	547	1558	36.1	39.08	5.93	10.71
TS 4	399	1419	28.1	37.95	5.17	10.48
Coker 312	547	1617	34.6	38.34	6.55	11.08
DES 119	716	1683	41.5	39.05	5.85	10.98
LSD 0.05	212	222	14.3	0.70	0.27	0.58
F Test	*	NS	NS	*	*	*

† Percent of potential equals yield of W/TBW divided by the yield of WO/TBW.

Table 3. Fiber properties of the transgenic lines grown under field conditions.

Cotton Strains	Fiber micronaire	Fiber length		Fiber elongation	Fiber strength
		50% span	2.5% span		
		----------mm----------		----%-----	-knm/kg-
TS 1	4.31	15.17	31.61	7.50	200.00
TS 2	4.15	15.23	32.18	7.35	209.71
TS 3	4.37	15.29	31.53	7.40	206.58
TS 4	4.26	15.43	32.12	7.27	205.96
Coker 312	4.15	15.21	31.45	7.25	203.67
DES 119	4.54	15.01	30.16	8.63	202.00
LSD 0.05	0.23	0.46	0.71	0.34	7.53
F Test	*	NS	*	*	NS